GENETIC DIFFERENTIATION OF ARGALI SHEEP *OVIS AMMON* IN MONGOLIA REVEALED BY MITOCHONDRIAL CONTROL REGION AND NUCLEAR MICROSATELLITES ANALYSES

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The genetic distinctiveness and possible gene flow among the Argali Sheep (*Ovis ammon*) populations in Mongolia have been controversial, due to a high degree of morphological variation among populations and an apparent lack of physical barriers to dispersal. We studied the population genetic structure of Argali sheep in Mongolia using both mitochondrial control region sequences (613 bp) and 14 nuclear microsatellite markers. Mitochondrial results suggest two evolutionarily distinct lineages, one in the Altay Mountains and the other in the Hangay Mountains and eastern Gobi Desert. Microsatellite analysis indicated genetic differentiation among these three regions, and also indicated similar levels of genetic differentiation and gene flow among all pair-wise comparisons. These results suggest genetic differentiation among the Mongolian populations of this endangered mammal.

Keywords: Argali sheep, mitochondrial DNA, microsatellites, *Ovis ammon*, population genetic structure

INTRODUCTION

The Argali Sheep (*Ovis ammon*), which is the largest species of wild sheep in the world, has become endangered due to poaching and habitat destruction (Valdez 1982; U.S. Fish and Wildlife Service 1996). Currently in Mongolia, Argali Sheep are patchily distributed in the Altay Mountains, Hangay Mountains, and Gobi Desert (Mallon 1985). Following a country-wide survey in 2002, Frisina *et al.* (2007) estimated a Mongolian Argali population of about 20,000. Across this range, both elevation and habitat productivity decrease gradually without obvious physical barriers to dispersal (Frisina 1998). Morphologically, Mongolian Argali Sheep is highly variable, and there is a general trend for average body size to decrease as elevation decreases from west to east, with Altay Argali being the largest in body size of all *O. ammon* (Geist 1991).

The variable morphology and the lack of obvious geographic barriers to gene flow have led to a long-debated controversy regarding the taxonomic status of Mongolian Argali and the delineation of genetically distinct populations. Allen (1940) considered all Mongolian Argali to be one subspecies *O.a. darwini*, but currently two subspecies are commonly recognized: *O.a. ammon* (Altay Argali) are large argalis from the Altay mountain region, and *O.a. darwini* (Gobi Argali) are smaller argalis from Gobi desert region (Sopin 1982; Valdez 1982; Geist 1991; Mitchell and Frisina 2007). Detailed analysis of cranial morphology show that *O.a. ammon* and *O.a. darwini* are morphologically distinct from other argalis and from each other, thus supporting subspecific recognition of these taxa (Kapitanova *et al.* 2004). The taxonomic position of argali from the Hangay region of Mongolia is unclear: Sopin (1982) and Geist (1991) considered these argalis to be similar to those from Altay (*O.a. ammon*), but genetic analyses by Tserenbataa *et al.* (2004) suggest that they may be more closely allied to Gobi argalis (see below).

Although a species of conservation concern, at present little is known about population structure and gene flow among argali populations in Mongolia. Tserenbataa *et al.* (2004) examined genetic variation at the mitochondrial ND5 locus, and found little genetic differentiation among populations in Mongolia and nearby regions of Kazakhstan and Kyrgyzstan. What little genetic differentiation they did find appeared to separate Gobi/Hangay from Altay/Kazakhstan/Kyrgyszstan. Tserenbataa *et al.* (2004) attributed the lack of genetic differentiation to high levels of female-mediated gene flow among populations, and concluded that argali populations from all of Mongolia and nearby regions of China and Russia should be considered a single "evolutionary significant unit" (or subspecies) with two management units.

The conclusions of Tserenbataa *et al.* (2004) contrasts with those from morphometric analyses, which suggest two argali subspecies in Mongolia (Kapitanova *et al.* 2004). Moreover, the shallow ND5 phylogenetic tree presented by Tserenbataa *et al.* (2004) suggests that the lack of genetic differentiation among populations may be due to incomplete lineage sorting rather than to the movement of individuals

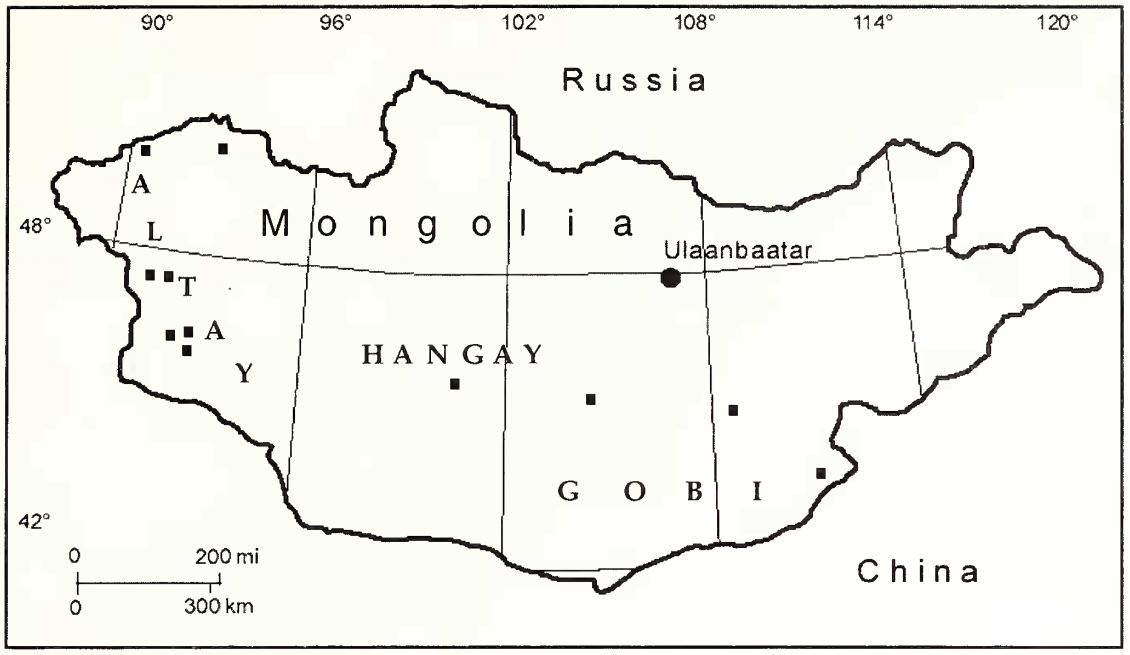

Fig. 1: Geographic distribution of sampling locations (filled squares) in Mongolia

(Avise 2000). To help resolve this taxonomic controversy and aid in conservation efforts, we studied two molecular genetic markers that show relatively high evolutionary rates – the mitochondrial control region and nuclear microsatellites – for argali samples collected in Mongolia.

Abbreviations: mtDNA = mitochondrial DNA, MP = maximum parsimony, ML = maximum likelihood, ME = minimum evolution

MATERIAL AND METHODS

We collected a total of 58 argali samples from Altay Mountains, Hangay Mountains and eastern Gobi Desert in Mongolia (Fig. 1), including tissue samples (skin and liver) collected from legally hunted individuals and bone samples (horn fragments and teeth) collected from carcasses found in the field. Skin samples of Snow Sheep (*Ovis nivicola*) from Russia were collected to serve as an outgroup for phylogenetic comparisons. Despite considerable and repeated efforts to extract and amplify DNA from all sources, some sources (e.g., some bone samples from carcasses) proved difficult and did not yield usable DNA. Consequently, the sample sizes for mtDNA and microsatellite analyses (see below) differ from each other and from the total number of samples collected.

We used a standard proteinase K digestion and phenol/chloroform methods to extract genomic DNA (Sambrook *et al.* 1989). The mtDNA control region was amplified via polymerase chain reaction (PCR) using primers modified for ungulates (Murray *et al.* 1995), and sequenced the portion proximal to $tRNA^{PRO}$ via cycle sequencing (Feng *et al.* 2001). Replicate amplifications were sequenced for most samples, and replicates always yielded identical results. Moreover, all sequences were clean and easily scored, suggesting that we did not co-amplify nuclear paralogs or encounter heteroplasmy. All haplotype sequences have been deposited in Genbank (accession numbers AY315886-AY215899). We used maximum parsimony, maximum likelihood, and minimum evolution approaches for phylogenetic analyses of the control region haplotypes (details given below). These analyses were conducted using PAUP*4.0b2 (Swofford 1998).

We screened 37 pairs of primers of dinucleotide-repeat microsatellites developed from domestic sheep (Crawford *et al.* 1995) in 10 argali individuals, and found 14 loci to be polymorphic: ILS5, ILS56, MAF33, MAF36, MAF48, MAF64, MAF209, FCB128, FCB226, FCB304, OHH35, OHH56, OVH72, and OVH116. Published primers and annealing temperatures (Crawford *et al.* 1995) to amplify alleles under the following conditions: 10 µl total reaction volume containing 20-50 µg genomic DNA, 1x PCR buffer (Roche), 0.05 mM dNTPs, 0.05 mM of each primer, 3.0 mM $MgCl_2$, 0.5U Taq polymerase, and 15 µCi ^{33}P-dATP (to label alleles). The PCR program was 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, annealing temperature for 1 min, and 72 °C for 45 sec. The last cycle was followed by a 5 min

extension at 72 °C. We separated PCR products by electrophoresis through a 6% polyacrylamide gel and ran a M13 sequence standard along with our PCR products for sizing the alleles. As with sequence analyses, replicates were amplified and electrophoresed for most samples to ensure accuracy of results.

For each population, the program GENEPOP web version 3.1c (Raymond and Rousset 1995a) was used to calculate genetic diversity for each locus, as well as the mean observed heterozygosity across all loci. Global tests of both allelic and genotypic distributions were performed to detect population differentiation. F_{st} (calculated using FSTAT 1.2, Goudet 1995) rather than R_{st} was used to measure population differentiation because the former performs better when sample size is small (Gaggiotti *et al.* 1999). The genetic distance calculator at http://www.biology.ualberta.ca was also used to calculate pair-wise Nei's genetic distances between populations. At the individual level, we conducted assignment tests (Paetkau *et al.* 1995; Waser and Strobeck 1998) using the calculator available at http://www.biology.ualberta.ca. We also calculated the pair-wise allele-sharing genetic distance (Bowcock *et al.* 1994) matrix, which was then subjected to PAUP*4.0b2 (Swofford 1998) and multidimensional scaling analysis in two dimensions (Manly 1997) with SPSS to test whether genetic similarity reflects geographic groupings.

RESULTS

Mitochondrial Control Region Phylogeny: We obtained 14 argali control region haplotypes from 17 sequences. We aligned 613 bp (including indels), of which 92 (15.0%) were variable (Fig. 2), and 32 of these were parsimony informative. The maximum likelihood estimate of transition/transversion ratio was 3.4:1. Base frequencies did not differ significantly across taxa ($\chi^2 = 23.07$, df = 45, p = 0.997), with A = 38.9%, C = 21.8%, G = 11.3%, and T = 28.0%. The sequence data contained significant phylogenetic signal as indicated by both a permutation test (PTP test, 1000 replicates, $p < 0.001$), and a tree length skewness test (*g1* test, 10,000 random trees, $p < 0.01$). Hierarchical likelihood ratio tests indicated that the optimal sequence evolution model for our observed data was the HKY+G model, which incorporates unequal base frequencies, unequal transition vs. transversion rates, and among site rate heterogeneity. The rate heterogeneity distribution parameter was $\alpha = 0.56$ (S.E. = 0.10), and the total heterogeneity (Gu *et al.* 1995) was 0.64.

Four maximum parsimony (MP) trees were found through a branch and bound search. Maximum likelihood (ML) and minimum evolution (ME) analyses yielded tree topologies that were concordant with the strict consensus MP tree (Fig. 3). The tree topology indicated that haplotypes from Hangay and east Gobi are more closely related to each other than they are to those from Altay. Seven out of eight haplotypes from Altay formed a single well-supported clade ("Altay group"). Haplotypes from Hangay and Gobi, plus a single haplotype from southern Altay, formed another well-supported clade ("Hangay/Gobi group"). Pair-wise ML genetic distance between the Altay group and Hangay/Gobi group (5.32% ±0.08%) was greater than that within the Altay (1.19% ±0.13%) and Hangay/Gobi groups (0.67% ±0.07%). Factoring out intra-group variation, the average ML genetic distance between the two groups was 4.39%.

Microsatellite Diversity and Differentiation: We obtained microsatellite genotype data at 14 loci for a total of

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          0000001111111111111111111111122222222222222222222233334444444444445555555555555555555556666
          0177991277777777778888999999990000000000113346777882356012233356889001226667778899999990011
          8325081501234567890123234567890123456789390431145678831338901752452237050182585891234579460 1
7M(A)     ATTTCGCTGCTCACATAACAACCCATACAGAAAAGCACAATCACTTAGGATGTCAATCGTTAC-TAACCCAGTAAAGTATAG-CATTTACCC
M3(A)     -CC..AT.....................................C.GTCCGAAGCACTG.CT-G.GTT.....T.....TAC.........A..A.
M6(A)     .CC..ATC....................................C.GTCCGAAG.ACTG.C...-GTT...T.......TAC.........-..A.
M7(A)     .CC..A......................................C.GTCCGAAG.ACTG.CT-G.GTT.....T...-.TAC.........-..A.
M9(A)     .CC..AT.....................................C.GTCCGAAG.ACTG.CT-G.GTT.........-.TAC...AG....A..A.
M19(A)    .CC.TA..-----------------------------------C.GTCCGAAG.ACTG.CT-G.G.T....TT.....TAC.........-T.A.
M20(A)    .CCC.A.....................................TGTCCGAA..ACTG.CT-G.GTTGT...T.....TAC.........AGAA.
M21(A)    .CC..A......................................C.GTCCGAA..ACTG.CT-G.GTT.....T.....TAC..T.....A..-.
M13(H)    .C.....................................................C.C..GC.-G..TT.-G....A.-.TA..C.....C.A..A.
M14(H)    .C.....................................................C.C..GC.-G..TT.-G...GA...TA..C.....C.A..A.
M15(H)    .C.....................................................C.C..GC.CG..TT..G....A.-.TA..C..CACC.A..A.
M17(H)    .C.....................................................C.C..GC.-G..TT.-G....AG..TA..C.....CGG..-.
M18(H)    .C.....................................................C.C..GC.-G..TT.-G...-A...TA.TC.....C.A...T
Arg2(G)   .C.....................................................C.C..GC.-G..TT..G....A..GTAC.......C.A..A.
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Fig. 2: Sequence alignment of polymorphic nucleotide sites for Argali control region haplotypes

Note: Numbers along the top refer to positions in the consensus sequence; Sample names are given in the extreme left column; and letters in parentheses refer to sampling location (A = Altay, H = Hangay, and G = Gobi); the top row gives the sequence for a reference sample (7M); dots indicate nucleotides that are identical to the reference; and dashes indicate deletions

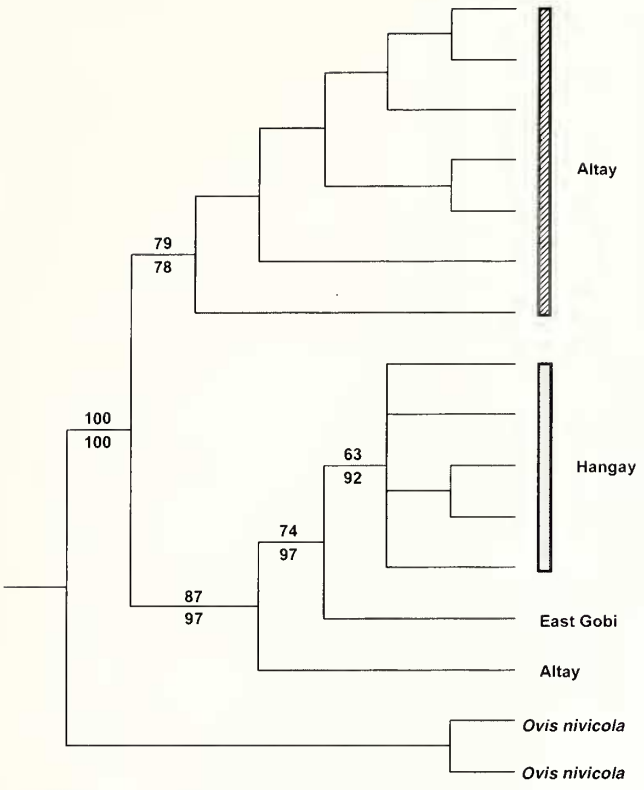

Fig. 3: Strict consensus tree of four most parsimony trees (tree length 343, CI = 0.904, RI = 0.907, RC = 0.820) rooted with snow sheep (*Ovis nivicola*). Transversion: transition ratio was 3:1, gaps were treated as a fifth state, and the branch and bound search algorithm was used. Numbers above the branches indicate the bootstrap values obtained through 1000 replications (only values above 50% are shown). The maximum likelihood tree had an identical topology, and numbers below branches indicate quartet puzzling support values (only values above 50% are shown)

30 individuals (12 from Altay, 11 from Hangay, and 7 from Gobi). There were no significant deviations from Hardy-Weinberg equilibrium (U tests, Raymond and Rousset 1995b), and no significant linkage disequilibrium (Fisher exact tests).

The total number of alleles for a locus across populations ranged from 4 to 13 (mean = 7.5, S.E. = 0.79), and observed heterozygosity ranged from 0.30 to 0.77 (mean = 0.61, S.E. = 0.04). For Altay, Hangay, and Gobi populations, the average number of alleles per locus (± S.E.) was 5.9 (±0.47), 5.0 (±0.47), and 3.6 (±0.36) respectively, and the mean observed heterozygosity was 0.61 (± 0.04), 0.65 (± 0.06), and 0.53 (± 0.07) respectively.

Fisher's exact test conducted on microsatellite allele and genotype frequencies showed significant population differentiation (P<0.001). The mean F_{st} for all loci was 0.056 (S.D. = 0.017), which was significantly greater than zero (permutation test with 1000 replications, P < 0.001). Pair-wise F_{st} values were similar across population pairs (range: 0.051 to 0.056), as were Nei's pair-wise genetic distances (range: 0.213 to 0.272). Likelihood assignment tests yielded a high percentage of correct assignments (26 of 30, 87%). For Gobi, all of 7 individuals were assigned to Gobi. For Altay, 10 of 12 individuals were assigned to Altay, 1 was assigned to Hangay, and 1 was assigned to Gobi. For Hangay, 9 of 11 individuals were assigned to Hangay, 1 was assigned to Altay, and 1 was assigned to Gobi. The percentage of correct assignment was significantly higher than expected by chance ($\chi^2 = 25.6$, df = 1, $p < 0.005$), suggesting significant differences in genotype frequencies among examined populations. However, the allele-sharing genetic distance matrix failed to generate geographic clustering of individuals, and multi-dimensional scaling showed a poor fit of data into two dimensions (Stress = 0.30, $R^2 = 0.53$).

DISCUSSION

Genetic Differentiation of Argalis in Mongolia: Due to a lack of apparent topographic boundaries and yet highly variable morphology across populations, controversy surrounds the level of genetic differentiation among Mongolian argali populations (Allen 1940; Sopin 1982; Valdez 1982; Geist 1991). This controversy has continued in large part due to the difficulties of obtaining genetic samples from the remote range of this species, and these difficulties also limited the sample size of our own analyses. Nevertheless, despite the limited sample size, our analyses of both mtDNA control region and nuclear microsatellites revealed significant genetic differentiation among sampled argali populations in Mongolia.

The mtDNA control region phylogeny revealed two major groups – one composed of individuals from Altay and the other comprised primarily of individuals from Hangay/Gobi – with an average sequence divergence (4.39%) similar to that observed between subspecies in other large mammals (Douzery and Randi 1997; Wooding and Ward 1997; Arctander *et al.* 1999; Matsuhashi *et al.* 1999), and greater than that typically seen among populations of Bighorn Sheep (*Ovis canadensis*; Ramey 1995; Luikart and Allendorf 1996; Boyce *et al.* 1999). This is also consistent with the speculation that habitat differences and a subtle geographic barrier (the Alakhnur Depression) have led to a reproductive isolation between Argali Sheep living in the Altay Mountains and those living in the Gobi desert (Sopin 1982). Interestingly, one Altay haplotype grouped with, but was basal to, the Gobi/Hangay group. Though this might be due to a low level of gene flow between the regions, incomplete lineage sorting seems a more plausible explanation.

Within the Hangay/Gobi group, all Hangay haplotypes formed one monophyletic subgroup and the single Gobi haplotype was positioned outside this Hangay group. Although this suggests some differentiation between Hangay and Gobi, additional samples from Gobi are required to further address the phylogenetic relationship between these populations.

Our nuclear microsatellite results also indicated significant genetic differentiation among the sampled populations. Although our sample sizes were small, the observed F_{st} value of 0.056 is similar to that found among natural populations of other large mammals (Roy *et al.* 1994; Forbes and Hogg 1999; Paetkau *et al.* 1999; Gutiérrez-Espeleta *et al.* 2000). Moreover, although the individual allele-sharing genetic distance matrix did not generate meaningful geographic groupings (probably due to small sample size) the high percentages of "correct" assignments yielded in the likelihood assignment test suggest that allele frequency distributions differ among the three populations. Furthermore, the similar pair-wise F_{st} and genetic distances and the even distribution of unassigned individuals suggest that these three populations are approximately equally differentiated from each other.

Implications for Argali Taxonomy: Currently, two subspecies of argali are commonly recognized in Mongolia (Sopin 1982; Valdez 1982; Geist 1991): *O.a. ammon* (Altay mountain region) and *O.a. darwini* (Gobi desert region). These subspecific designations are supported by morphometric analyses (Kapitanova *et al.* 2004), and our mitochondrial control region phylogeny supports the distinction between argali from these regions. In contrast, although Hangay argalis are currently classified as *O.a. ammon* based on morphological similarities (Sopin 1982; Geist 1991), our mitochondrial analyses instead suggests that Hangay argalis are more closely related to *O.a. darwini* than to *O.a. ammon* (Tserenbataa *et al.* 2004).

There are two possible explanations for this discrepancy. First, the more ammon-like morphology of Hangay argali may be due to the higher habitat productivity of the Hangay region relative to the arid Gobi desert. Second, because the mtDNA phylogeny only represents maternal descent, Hangay argali may be a hybrid form resulting from matings between large-bodied *ammon* males and small-bodied *darwini* females. This 'hybrid origin' hypothesis also can explain the approximately equal genetic distances that we obtained from nuclear microsatellite data. One way to test this hypothesis is to use a Y-linked marker to reconstruct the paternal lineage of Mongolian argalis. Moreover, since we were able to obtain only one sequence from Gobi argali, it is possible that the Hangay and Gobi populations represent two distinct subspecies of argali; this possibility requires further testing with additional haplotypes.

Implications for Conservation Management: Our mtDNA results showed that Mongolian argali haplotypes can be divided into two reciprocally monophyletic groups – one consisting of haplotypes found only in the Altay Mountains, and the other consisting almost exclusively of haplotypes from the Hangay Mountains and eastern Gobi desert. Due to the presence of one Altay haplotype in the Hangay/Gobi clade, argali in these two regions are not strictly reciprocally monophyletic, and therefore do not fit the definition of Evolutionary Significant Units (ESU's) suggested by Moritz (1994). Nevertheless, our mtDNA results suggest two distinct, independent lineages, and the ESU criterion suggested by Moritz (1994) has been criticized for being overly stringent (Crandall *et al.* 2000; Fraser and Bernatchez 2001). Therefore, we tentatively recommend that argali in Altay and Hangay/Gobi be treated as two separate ESU's for conservation purposes.

Our microsatellite analyses indicated significant nuclear genetic differentiation among all three regions of Mongolia. Our mitochondrial control region analyses also showed differentiation between our single Gobi haplotype and all Hangay haplotypes, with the latter forming a single monophyletic clade. These results suggest that each area should be treated as a separate management unit (Moritz 1994) for conservation purposes. However, this recommendation should be considered tentative because sample sizes and areas surveyed were limited in this study (particularly for mitochondrial analyses), and because microsatellites can sometimes show significant differentiation across populations that may not be biologically meaningful (Hedrick 1999).

Our results are mostly consistent with the mitochondrial ND5 analyses of Tserenbataa *et al.* (2004), who found significant genetic differentiation between the Altay and Hangay/Gobi regions. However, Tserenbataa *et al.* (2004) found that the differentiation between these two groups was relatively weak, that there was no significant differentiation between Hangay and Gobi populations, and that haplotypes from any region did not form a monophyletic group. Tserenbataa *et al.* (2004) concluded that there has been significant historical gene flow among the three regions of Mongolia and nearby areas of China and Russia, and that the entire region should be treated as a single ESU/subspecies. Our results indicate that the lack of resolution in the ND5 data of Tserenbataa *et al.* (2004) likely is due to the slow evolutionary rate of ND5 (compared to the control region and nuclear microsatellites) and incomplete lineage sorting rather than to the movement of individuals between regions. Nevertheless, studies that combine large sample sizes (as in Tserenbataa *et al.* 2004) with more sensitive genetic markers (as in this study) are needed before making firm conclusions about conservation units for Mongolian and other argali.

ACKNOWLEDGEMENTS

We thank D. Taylor, M.A. Coffroth, and C. Larsen for their insightful comments. We also gratefully acknowledge Grand Slam Club/Ovis, Ministry for Nature and the Environment of Mongolia, Juulchin Corporation, Mongol Tours, and the State University of New York for supporting the project. All samples used for this study were collected and imported under proper permits from Mongolia and the United States.

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